Regulation of Human B-Cell Precursor Adhesion to Bone Marrow Stromal Cells by Cytokines That Exert Opposing Effects on the Expression of Vascular Cell Adhesion Molecule-1 (VCAM-1)

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Self-renewal and differentiation of B-cell precursors is dependent on interactions with bone marrow (BM) stromal cells and associated extracellular matrix. We have recently developed an interleukin (IL)-7–dependent, BM-derived stromal cell culture that supports the growth of normal human B-cell precursors. In the current study, we have characterized the constitutive expression, cytokine-regulated expression, and function of adhesion molecules on BM stromal cells that are critical for adhesion of B-cell precursors. Flow cytometric analysis showed that cultured adult BM stromal cells expressed higher constitutive levels of vascular cell adhesion molecule (VCAM)-1 than intercellular adhesion molecule (ICAM)-1 (CD54). IL-1β upregulated VCAM-1 and CD54 in a dose-dependent manner, whereas IL-4 upregulated VCAM-1, but had no effect on CD54. In contrast, transforming growth factor (TGF)-β decreased the level of BM stromal cell VCAM-1. Using an assay to measure the adhesion of ^{51}Cr-labeled B-cell precursors to BM stromal cells, we observed a direct correlation between cytokine-regulated levels of VCAM-1 and the capacity of stromal cells to support the adhesion of B-cell precursors. Blocking studies using a panel of monoclonal antibodies (MoAb) showed that adhesion of B-cell precursors to untreated and cytokine-treated (IL-1β, IL-4) BM stromal cells was mediated by very late antigen (VLA)-4 (CD49d/CD29) and VCAM-1. Adhesion of B-cell precursors could also be enhanced by direct stimulation with MoAb to the CD29 subunit. Our collective results indicate that B-cell precursor/BM stromal cell adhesion is mediated by a VLA-4-VCAM-1 interaction, which in turn can be regulated at the level of the BM stromal cell by cytokines that specifically increase or decrease cell surface VCAM-1.

B LYMPHOPOIESIS in adult mammals occurs in the bone marrow (BM), and reflects the interaction of B-cell precursors with a BM microenvironment consisting of lymphohematopoietic cells, stromal cells, and the extracellular matrix. The continuous proliferation and differentiation of B-cell precursors at various stages of development is absolutely dependent on a functional stromal cell microenvironment, as shown by in vitro and in vivo studies in the mouse.[1,2] Recent studies in our laboratory have shown the capacity to grow normal human B-cell precursors for several weeks in vitro, using an interleukin-7 (IL-7)–dependent, BM-derived stromal cell culture system.[3]

A major area of focus in contemporary biology is the identification and characterization of cell surface molecules that mediate adhesive interactions between eukaryotic cells. Integrins are a widely expressed family of cell surface adhesion receptors.[4-6] All integrins consist of α/β noncovalently-associated heterodimers and are classified according to their β chain subunit.[4-6] Integrins have been implicated in cell-cell and cell-substratum interactions, with most cell types expressing at least one of about 20 distinct α/β integrin heterodimers.[4] The very late antigen (VLA) integrin subfamily contains at least eight distinct α chain members, all of which can associate with the β1 subunit.[4] Some members of the VLA subfamily have the capacity to bind more than one ligand. For example, VLA-4 (CD49d/CD29) binds to vascular cell adhesion molecule (VCAM)-1,[7-9] a member of the Ig superfamily first described as a cytokine-inducible molecule on endothelial cells.[10-12] VCAM-1 mRNA can undergo alternate splicing to give rise to a 6-Ig domain form and a 7-Ig domain form.[13,14] The 7-Ig domain form is the predominant form induced on endothelial cells, and contains a fourth domain that is absent from the 6-Ig domain form. Both the 6-Ig and 7-Ig forms can bind VLA-4, and VLA-4 binding sites exist within the first and fourth VCAM-1 domains.[15,16] VLA-4 can also bind to the alternatively spliced CS-1 domain of fibronectin (FN) via the FN sequence LDV,[17-19] whereas VLA-5 (CD49e/CD29) in hematopoietic cells binds FN via the RGD-containing central cell binding domain.[4,19] VLA-4 is expressed on murine[20] and adult human BM B-cell precursors,[21] and participates in the adhesion of B-cell precursors to BM stromal cells in both species.[20,21] Adhesion of activated B cells and follicular non-Hodgkin's lymphoma cells to germinal centers is mediated by VLA-4,[22-24] as is the adhesion of CD34$^+$ hematopoietic precursors to BM stromal cells.[25,26]

The leukocyte integrin lymphocyte function associated antigen (LFA)-1 is also expressed by B-cell precursors at a low, but detectable level.[21,27] LFA-1 is a member of the β2 family of integrins and has three known cellular ligands: intercellular adhesion molecule (ICAM)-1 (CD54),[28] ICAM-2,[29] and ICAM-3,[30] all members of the Ig superfamily. CD54 has been detected on BM fibroblasts by immunofluorescence microscopy.[21]

Cell–cell interactions mediated by integrins in vitro can be regulated by altering the activation state of the integrin using phorbol esters or monoclonal antibodies (MoAb) specific for the β subunit. For example, MoAb specific for the β1 (CD29) subunit have been described that enhance rather than inhibit cell–cell adhesion.[31-33] The favored explanation

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for these results is that the MoAb induces a conformational change in the integrin that "exposes" the ligand binding site, thereby enhancing adhesion.[4]

Our IL-7–dependent, BM-derived stromal cell culture system for growing normal human B-cell precursors is characterized by the close apposition of B-cell precursors to the BM stromal cells.[3] The goal of the current study was to define the expression and function of molecules on the B-cell precursors and BM stromal cells that mediate adhesion in this culture system. Our collective results indicate that B-cell precursor/BM stromal cell adhesion is mediated by a VLA-4/VCAM-1 interaction that can be regulated by cytokines with opposing effects on the expression of BM stromal cell VCAM-1.

MATERIALS AND METHODS

Cells. Bone marrow from normal 19- to 21-week-old human fetuses and normal adult donors was obtained in accordance with the guidelines of the University of Minnesota Committee on the Use of Human Subjects in Research. Femoral and humeral bones were scraped free of connective tissue and fasciae and flushed with RPMI 1640/2% fetal calf serum (FCS) (Hyclone, Ogden, UT) containing 100 U/mL penicillin and 100 μg/mL streptomycin. The bones were then minced into fine pieces, and the additional cells were collected. These were combined with the cells obtained from the initial flushing and centrifuged on a Ficoll-Hypaque gradient (Histopaque; Sigma Chemical Co. St Louis, MO). The interface cells were washed twice in RPMI 1640/2% FCS. Adherent cells were depleted by plastic adherence for 2 hours at 37°C in RPMI 1640/10% FCS. $CD10^+$/surface IgM^- B-cell precursors were isolated by magnetic bead depletion, as previously described.[34] The $CD10^+$/surface IgM^- population contained a mixture of approximately one third pro-B (cytoplasmic IgM^-) and two thirds pre-B (cytoplasmic IgM^+) cells, and was typically more than 90% pure.

Normal adult BM was centrifuged on a Ficoll-Hypaque gradient, the interface cells were washed twice in RPMI 1640/2% FCS, and 10 to 40 × 10^6 cells were seeded into 75-cm^2 culture flasks in EX-CELL 320 (JRH Biosciences, Lenexa, KS) containing 10% FCS, 100 U/mL penicillin, and 100 μg/mL streptomycin. Nonadherent cells were washed off after 1 week. On reaching confluency, the adherent BM stromal cells were switched to EX-CELL 320/0% FCS to maintain cell viability and to inhibit further proliferation. These adult BM stromal cells can remain viable for up to 7 months in EX-CELL 320/0% FCS with little detectable growth, and no change in function or morphology.

Antibodies and cytokines. The adhesion molecules analyzed with MoAb in the current study have several different designations. For purposes of continuity, we will use the CD nomenclature throughout. The exception is VCAM-1, which has not been assigned a CD number in the workshops held to date. Therefore, ICAM-1 = CD54, the α4 subunit of VLA-4 = CD49d, the α5 subunit of VLA-5 = CD49e, the β1 subunit of VLA-4 and VLA-5 = CD29, the α subunit of LFA-1 = CD11a, and the β2 subunit of LFA-1 = CD18. The CD49d/CD29 and CD49e/CD29 heterodimers will be referred to by their conventional acronyms VLA-4 and VLA-5, respectively.

4B9/anti-VCAM-1[35] and LB2/anti-CD54[36] were kind gifts from Dr John Harlan and Dr Ed Clark (University of Washington, Seattle, WA), respectively. P5D2 and P4F7/anti-CD29 (CD29 blocking MoAb), P4G11/anti-CD29 (CD29 activating MoAb), P4C2/anti-CD49d (blocks VLA-4 binding to both VCAM-1 and the CS-1 domain of FN), P4G9/anti-CD49d (blocks VLA-4 binding to the CS-1 domain of FN only), P1D6/anti-CD49e and P4H9/anti-CD18 (inhibits homotypic B-cell adhesion and HLA-restricted cytotoxic T-cell activity) were produced and characterized as described.[19,32,37,38] Hybridoma cells secreting W6/32/anti-HLA-A, B, C; OKM1/anti-CD11b and L243/anti-MHC class II were obtained from the American Type Culture Collection (Rockville, MD). The STRO-1 hybridoma[39] was obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD and the Department of Biology, University of Iowa, Iowa City, IA, under contract N01-HD-6-2915 from the National Institute of Child Health and Human Development. J5[40]/anti-CD10-phycoerythrin (PE), J5[40]/anti-CD10 and IgG2a-PE control myeloma protein were purchased from Coulter Immunology (Hialeah, FL). TA-1/anti-CD11a was produced and characterized in this laboratory.[41] Rabbit anti-human von Willebrand Factor (vWF) (Sigma) was a kind gift of Dr Greg Vercellotti (University of Minnesota, Minneapolis, MN). Rabbit gamma globulin was purchased from Jackson ImmunoResearch Laboratories, Inc (West Grove, PA). IgG1 and IgG2a control mouse myeloma proteins were purchased from Organon Teknika-Cappel (Durham, NC). Mouse myeloma IgG1 was conjugated to fluorescein isothiocyanate (FITC) by standard methods.[42] $F(ab')_2$ goat anti-human kappa-FITC, $F(ab')_2$ goat anti-human lambda-PE, $F(ab')_2$ goat Ig conjugated to FITC and PE, $F(ab')_2$ goat anti-mouse Ig-FITC and $F(ab')_2$ goat anti-rabbit Ig-FITC were purchased from Tago, Inc (Burlingame, CA). HPCA-2-FITC/anti-CD34 was purchased from Becton Dickinson Immunocytometry Systems (Mountain View, CA). FITC-goat anti-mouse IgG was purchased from Southern Biotechnology Associates, Inc (Birmingham, AL).

Recombinant human IL-1β, recombinant human IL-3, recombinant human IL-4, recombinant human IL-6, porcine transforming growth factor-β (TGF-β), and recombinant human basic fibroblast growth factor (FGF) were obtained from R & D Systems (Minneapolis, MN). Recombinant human IL-7 was obtained from PeproTech Inc (Rocky Hill, NJ). Recombinant human interferon-γ (IFN-γ) was a kind gift of Dr Phil McGlave (University of Minnesota, Minneapolis, MN). Lipopolysaccharide was a kind gift of Susan Christian (University of Minnesota, Minneapolis, MN).

Flow cytometry. Expression of cell surface molecules on BM stromal cells was analyzed by indirect immunofluorescence staining and flow cytometry. First- or second-passage adult BM stromal cells were removed from tissue culture flasks by a 5 to 10 minute incubation in trypsin-EDTA (GIBCO Laboratories Life Technologies, Inc, Grand Island, NY) at 37°C, washed once, and plated at 1 to 2 × 10^5 cells in 25-cm^2 tissue culture flasks containing EX-CELL 320/10% FCS. After 2 to 3 days the medium was changed to EX-CELL 320/0% FCS. The influence of cytokines on VCAM-1 and CD54 expression was studied by incubating BM stromal cells with varying doses of IL-1β, IL-4, or TGF-β diluted in EX-CELL 320/0% FCS for various lengths of time before analysis. Adult BM stromal cells were dissociated from tissue culture flasks with Cell Dissociation Solution (Sigma), washed once, and incubated on ice with 4B9 (anti-VCAM-1), LB2 (anti-CD54), L243 (anti-MHC Class II), J5 (anti-CD10), OKM1 (anti-CD11b) ascites, STRO-1 culture supernatant, or with IgM, IgG1, IgG2a myeloma proteins or control ascites, all used at saturated binding conditions. The cells were washed twice and counterstained with $F(ab')_2$ goat anti-mouse Ig-FITC (1:40) for 30 minutes on ice. After washing twice, the BM stromal cells were fixed in 1% paraformaldehyde and analyzed using CONSORT 30 software on a FACScan (Becton Dickinson). BM stromal cells were also stained for vWF as described above using rabbit anti-human vWF (10 μg/mL) or normal rabbit gamma globulin (10 μg/mL) as a control, followed by counterstaining with $F(ab')_2$ goat anti-rabbit Ig-FITC (1:40). Data are reported as the mean channel fluorescence (MCF) of 5,000 cells analyzed on a log scale.

Indirect immunofluorescence staining was used to examine the expression of CD54, CD11a, CD18, CD29, CD49d, and CD49e on B-cell precursors. Saturating amounts of LB2, TA-1, P4H9, P5D2, P4C2, and P1D6 were added to 5 × 10^5 cells for 30 minutes on ice. The cells were washed twice, counterstained with FITC-goat anti-

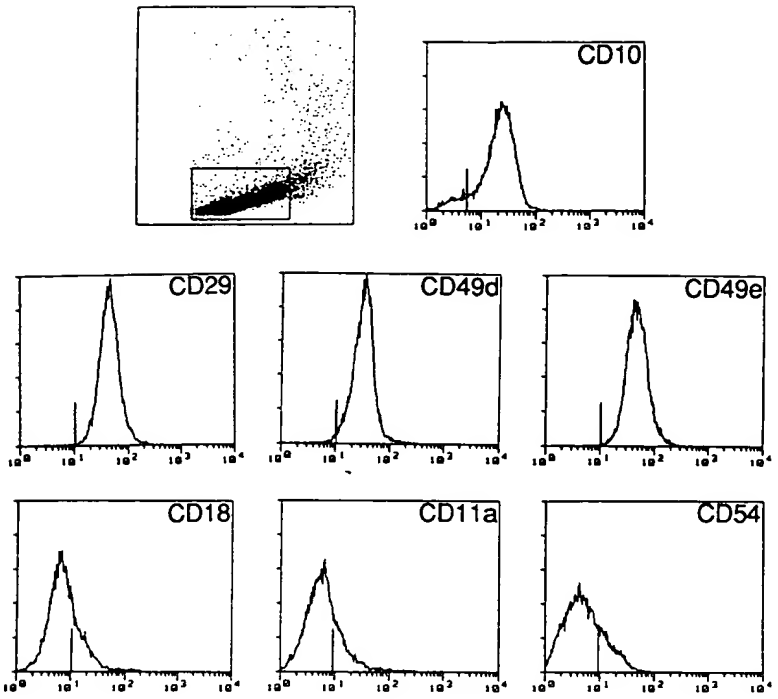

Fig 1. Expression of CD10, CD11a, CD18, CD29, CD49d, CD49e, and CD54 on human fetal BM cells with lymphoid light scatter characteristics (low to medium forward angle on the horizontal axis/ low 90° angle on the vertical axis, as defined by the gated area in the light scatter profile). Histograms represent fluorescence intensity on the horizontal axis and relative cell number on the vertical axis. The vertical bar in each histogram defines background staining, with greater than 95% of events falling to the left of the vertical bar.

mouse IgG (1:100) for 30 minutes, and analyzed on a FACScan as described above. Negative controls consisted of cells incubated with identical concentrations of control ascites, IgG2a, or IgG1. CD10 expression was detected by a similar method using saturating amounts of J5-PE and IgG2a-PE as a negative control.

Adhesion assay. $CD10^+$/surface IgM^- B-cell precursors were radiolabeled by incubating 10 to 20 × 10^6 cells with 100 to 200 μCi $Na_2{}^{51}CrO_4$ for 2 hours at 37°C. The B-cell precursors were washed twice and resuspended in EX-CELL 320/0% FCS. ^{51}Cr-labeled B-cell precursors typically had 6,000 to 10,000 cpm/50,000 cells.

BM stromal cells were seeded onto 96-well flat-bottom tissue culture treated strip-well microtiter plates (Costar, Cambridge, MA), at 2,000 to 3,000 cells per well in EX-CELL 320/10% FCS. After 2 to 3 days the medium was changed to EX-CELL 320/0% FCS and maintained until further use (3 to 4 days). Before the addition of ^{51}Cr-labeled B-cell precursors, the BM stromal cells were incubated with or without IL-1β (10 pg/mL), IL-4 (10 ng/mL), or TGF-β (10 ng/mL). After 18 to 24 hours the BM stromal cells were washed twice with EX-CELL 320/0% FCS, and saturating concentrations of blocking antibodies and the anti-CD29 activating MoAb P4G11 diluted in EX-CELL 320/0% FCS were added. ^{51}Cr-labeled B-cell precursors, 5 × 10^4, were immediately added to the BM stromal cells and incubated at 37°C for 2 hours. Nonadherent cells were removed by washing twice using a 12-well multichannel pipettor (Costar). The first wash consisted of removing ~¾ of the medium from the wells, redispensing the medium down the sides of the wells, and then removing all the medium. After a second wash with 200 μL fresh EX-CELL 320/0% FCS, the adherent B-cell precursors were quantitated by breaking apart the stripwells and placing each well in an individual glass tube. Bound counts per minute (cpm), corresponding to bound cells, was quantitated on a γ-counter for 1 minute. Each experimental variable was tested in replicates of eight. Percent B-cell precursor adherence was calculated by the formula:

$$\% \text{ Adherence} = \frac{\overline{X} \text{ Adherent cpm}}{\text{Total Input cpm}} \times 100.$$

Percent adherence of B-cell precursors to BM stromal cells ranged from 13% to 44% adhesion in 14 independent experiments. First- or second-passage BM stromal cells were used in the adhesion assays to assure optional conditions encompassing stromal cell spreading and strong VCAM-1 expression. Human skin fibroblasts passaged more than twice develop a "dendritic-like" morphology, become postmitotic, and degenerate.[43] We have noticed similar characteristics in our BM stromal cell cultures. BM stromal cells were maintained in culture for greater than 4 months without loss of VCAM-1 expression. Statistical significance was determined by the unpaired Student's *t*-test using Statworks (Cricket Software, Philadelphia, PA).

Phenotypic analysis of adherent and nonadherent B-cell precursors. Adherent and nonadherent B-cell precursors were isolated by incubating mock ^{51}Cr-labeled B-cell precursors with BM stromal cells and washing, as described above. The nonadherent cells from 96 wells were pooled, and the adherent cells were subsequently collected by incubating the washed stromal cells with 200 μL Cell Dissociation Solution, followed by removal of adherent B-cell precursors with vigorous pipetting. Preadherent, adherent, and nonadherent B-cell precursors were analyzed for the expression of CD34 and surface Ig light chains using the immunofluorescent staining method described above.

RESULTS

Expression of adhesion molecules on fetal BM B-cell precursors. Previous studies have described the expression of adhesion molecules on B-cell precursors in adult BM.[21,27] Because human adult and fetal BM B-cell precursors exhibit differences in cell surface antigen expression,[44] we analyzed fetal BM B-cell precursors for expression of adhesion molecules. As shown in Fig 1, essentially all $CD10^+$ fetal BM B-cell precursors express CD29, CD49d, and CD49e. In contrast, CD18, CD11a, and CD54 are weakly expressed on $CD10^+$ fetal BM B-cell precursors.

Phenotypic analysis of BM stromal cells. BM stromal cells were studied for expression of cell surface molecules by flow cytometry. As shown in Fig 2, BM stromal cells from a 35-day culture (passaged twice) expressed VCAM-1 and CD10,

did not express CD11b, and expressed very low MHC class II. The absence of CD11b and very low MHC class II indicates that few, if any, monocyte/macrophages were present. STRO-1 is an antigen expressed on BM stromal cells grown under Dexter culture conditions.[39] As shown in Fig 2, BM stromal cells were STRO-1$^-$, as were BM stromal cells from three other donors. The activity of the STRO-1 MoAb was confirmed by showing binding to glycophorin A$^+$ erythroid precursors (data not shown), as previously described.[39] Interestingly, a distinct subpopulation of BM stromal cells (22%) expressed vWF (Fig 2), suggesting the presence of BM-derived endothelial cells. This subpopulation of vWF$^+$ cells may reflect the age of the culture because separate 56 day, 125 day, and 130 day cultures from different donors contained 20% vWF$^+$, 5% vWF$^+$, and 4% vWF$^+$ cells, respectively.

Cytokine regulation of VCAM-1 and CD54 on BM stromal cells. The expression of endothelial cell surface VCAM-1 and CD54 is regulated by cytokines.[8,10,11,35] Therefore, we determined whether BM stromal cell VCAM-1 and CD54 were regulated in a similar manner. Initial studies showed that VCAM-1 (Fig 3A) is constitutively expressed at a higher cell surface level than CD54 (Fig 3E) on BM stromal cells. BM stromal cells from 11 other donors showed a similar expression of VCAM-1 and CD54. The ability of selected cytokines to modulate BM stromal cell surface expression of VCAM-1 and CD54 was then examined. Incubation of BM stromal cells with 1 to 100 pg/mL IL-1β induced a subtle dose-dependent upregulation of VCAM-1 (Fig 3B) and a more pronounced dose-dependent upregulation of CD54 (Fig 3F). IL-1β upregulation of VCAM-1 reached maximal levels at 10 pg/mL, whereas CD54 expression increased dose-dependently up to 100 pg/mL with an additional increase also detected at 1,000 pg/mL (data not shown). VCAM-1 upregulation was marginally detected at 0.1 pg/mL IL-1β (data not shown), whereas CD54 upregulation was detected at 1.0 pg/mL. Similar to the effect of IL-1β, 0.1 to 10.0 ng/mL IL-4 upregulated VCAM-1 (Fig 3C) expression in a subtle dose-dependent manner. In contrast, CD54 was not upregulated with this concentration range of IL-4 (Fig 3G). VCAM-1 upregulation was detected at 0.1 ng/mL IL-4 (Fig 3C), but 50 ng/mL IL-4 was required to induce a detectable increase in CD54 expression (data not shown).

In contrast to the upregulating effects of IL-1β and IL-4 on BM stromal cell VCAM-1 expression, TGF-β exerted a dose-dependent negative regulatory effect on the expression of VCAM-1 (Fig 3D). TGF-β did not alter the expression of CD54 (Fig 3H), even at a TGF-β concentration of 10 ng/mL (data not shown). This was probably attributable to the low constitutive expression of CD54. IFN-γ (100 U/mL) and lipopolysaccharide also upregulated VCAM-1 and CD54 expression, whereas IL-3, IL-6, IL-7, and FGF had little or no effect (data not shown).

Time course analysis of cytokine regulation of VCAM-1 and CD54. Figure 4A shows a time course analysis of enhanced cell surface VCAM-1 expression by IL-1β (100 pg/mL) and IL-4 (10 ng/mL). The initial enhanced expression of VCAM-1 by IL-1β and IL-4 were similar: a slight increase was detectable at 2 hours, and parallel increases occurred up to 12 hours. IL-1β enhanced expression decayed after 12 hours, and returned to approximately control levels by 24 hours. In contrast, IL-4 enhanced expression plateaued at 18 to 24 hours (Fig 4), and remained elevated for several days (data not shown). Figure 4B shows that IL-1β enhanced expression of CD54 was detectable at 2 hours, plateaued at 12 hours, and remained elevated for several days (data not shown). IL-4 at 10 ng/mL did not increase the expression of CD54 over the 24-hour time course. Figure 4C shows the kinetics of TGF-β (1 ng/mL) downregulation of VCAM-1 expression on BM stromal cells. The decrease in VCAM-1 expression was first detectable between 4 and 8 hours and gradually decreased up to 24 hours.

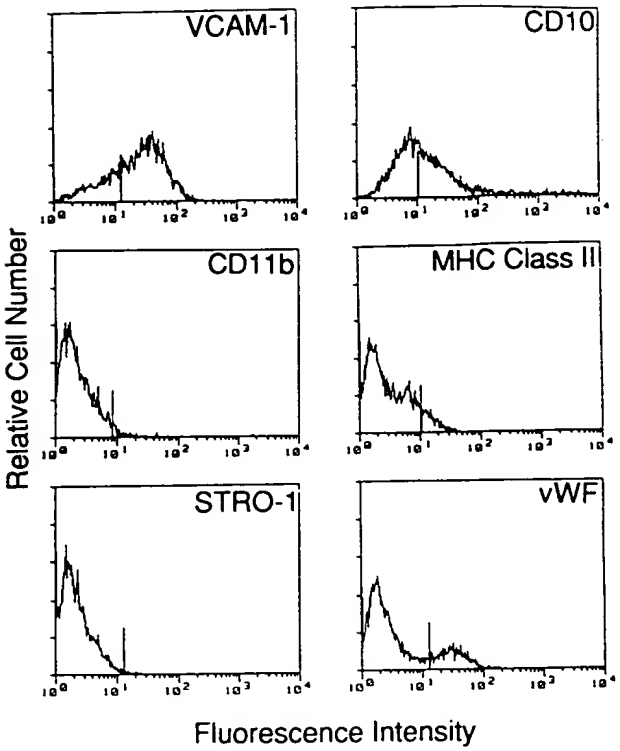

Fig 2. Expression of VCAM-1, CD10, CD11b, MHC Class II, STRO-1, and vWF on a 35-day culture (passaged twice) of BM stromal cells. The vertical bar in each histogram defines background staining, with greater than 95% of events falling to the left of the vertical bar.

Adhesion of B-cell precursors to BM stromal cells parallels the cytokine-regulated expression of VCAM-1. We have recently shown that growth of human B-cell precursors in vitro requires IL-7 and BM-derived stromal cells.[3] Therefore, we investigated the mechanism of adhesive interactions between BM stromal cells and B-cell precursors, and whether the adhesive interaction could be regulated by cytokines. We were particularly interested in determining whether B-cell precursor adherence to BM stromal cells was related to the level of expression and function of VCAM-1, and whether this could be regulated by IL-1β, IL-4, or TGF-β. Table 1 shows a representative experiment comparing the percent adhesion of ^{51}Cr-labeled CD10$^+$/surface IgM$^-$ B-cell precursors to untreated or cytokine-treated BM stromal cells, to the level of VCAM-1 and CD54 expression. Incubation of BM stromal cells with IL-1β (100 pg/mL) increased the mean channel

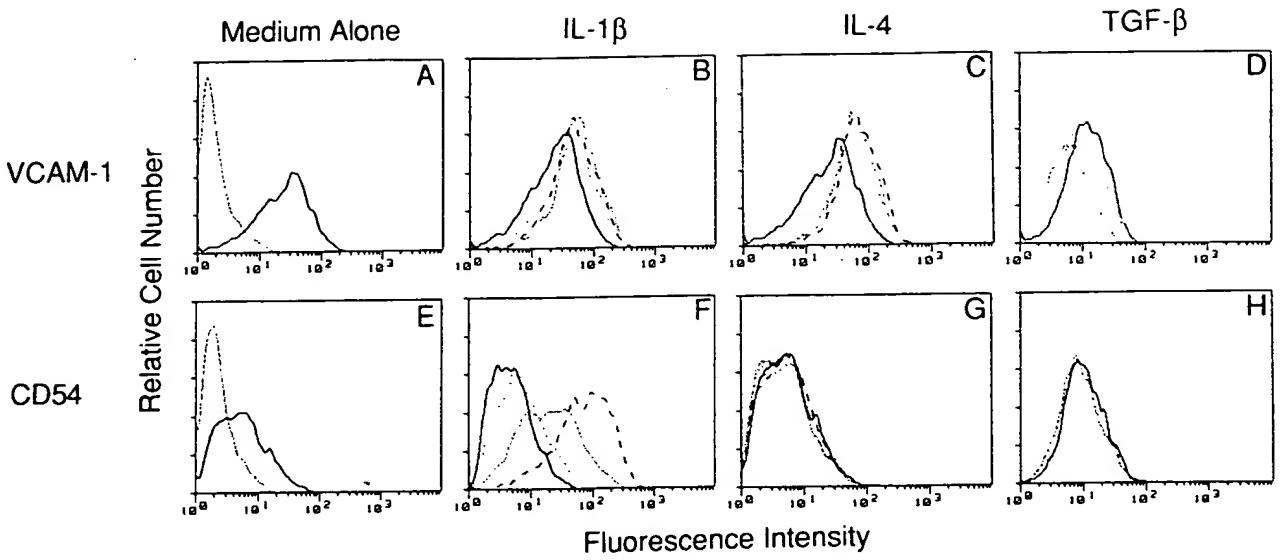

Fig 3. Dose-dependent regulation of BM stromal cell VCAM-1 and CD54 expression by IL-1β, IL-4, or TGF-β. BM stromal cells were incubated with (A, E) medium alone (EX-CELL 320/0% FCS) or medium containing varying concentrations of (B, F) IL-1β, (C, G) IL-4, or (D, H) TGF-β for 18 to 24 hours. Expression of VCAM-1 and CD54 was analyzed by indirect immunofluorescence on a FACScan. The MoAb 4B9 (IgG1) and LB2 (IgG2a) were used at 10 μg/mL to detect VCAM-1 and CD54, respectively. A-expression of VCAM-1 (——) (MCF = 35) and background staining with an IgG1 isotype-matched control (····) (MCF = 3); E-expression of CD54 (——) (MCF = 9) and background staining with an IgG2a isotype-matched control (····) (MCF = 3); B-expression of VCAM-1, without (——) (MCF = 35) or with 1 pg/mL (····) (MCF = 58), 10 pg/mL (····) (MCF = 72) or 100 pg/mL (– – – –) (MCF = 65) IL-1β; F-expression of CD54, without (——) (MCF = 7) or with 1 pg/mL (····) (MCF = 10), 10 pg/mL (····) (MCF = 34) or 100 pg/mL (----) (MCF 105) IL-1β; C-expression of VCAM-1 without (——) (MCF = 35) or with 0.1 ng/mL (····) (MCF = 72), 1 ng/mL (····) (MCF = 79) and 10 ng/mL (----) (MCF = 90) IL-4; G-expression of CD54 without (——) (MCF = 9) or with 0.1 ng/mL (····) (MCF = 8), 1 ng/mL (····) (MCF = 8) and 10 ng/mL (----) (MCF = 9) IL-4; D-expression of VCAM-1 without (——) (MCF = 15) or with 0.1 ng/mL (····) (MCF = 12) and 1 ng/mL (····) (MCF = 8) TGF-β; H-expression of CD54 without (——) (MCF = 14) or with 0.1 ng/mL (····) (MCF = 12) and 1 ng/mL (····) (MCF = 12) TGF-β. Background staining with isotype-matched controls for B, C, D, F, G, and H are not shown, but were similar to A and E. Histograms have been smoothed 10 times to facilitate resolution of the data. Histograms A, C, E, and G are from a single experiment, histograms B and F are from a single experiment, and histograms D and H are from separate experiments.

fluorescence of VCAM-1 from 26 to 53, and CD54 from 9 to 61. This increased expression of VCAM-1 and CD54 correlated with a significant increase in B-cell precursor adhesion from 19% to 26%, representing a 34% increase in adhesion. IL-4 (10 ng/mL) increased VCAM-1 expression on BM stromal cells from a mean channel fluorescence of 26 to 61, but had no influence on the expression of CD54. IL-4 treatment of BM stromal cells also correlated with a significant increase in B-cell precursor adhesion from 19% to 33%, representing a 72% increase in adhesion. In contrast, TGF-β (10 ng/mL) decreased the cell surface expression of VCAM-1 from a mean channel fluorescence of 26 to 15, leading to a significant decrease in B-cell precursor adhesion from 19% to 14%, representing a 27% decrease in adhesion.

Cytokine-regulated adhesion of B-cell precursors to human BM stromal cells occurs via a VLA-4-VCAM-1 interaction. The mechanism of B-cell precursor adhesion to untreated or cytokine-treated BM stromal cells was examined using a panel of blocking MoAb (Fig 5). Figure 5A shows one representative experiment demonstrating 33% ± 0.7% adhesion of B-cell precursors to untreated BM stromal cells, while adhesion to plastic alone (no stroma) was 4% ± 0.5%. B-cell precursor adhesion to BM stromal cells was shown to be mediated by a VLA-4/VCAM-1 interaction because MoAb directed against CD49d-FN + V (MoAb P4C2 blocks VLA-4 binding to both VCAM-1 and the CS-1 domain of FN) (inhibition = 75%) and CD29 (inhibition = 88%) significantly decreased adhesion. The VCAM-1 specific antibody 4B9 also significantly blocked B-cell precursor adhesion to BM stromal cells (inhibition = 52%), albeit not as effectively as the antibodies to CD49d-FN + V and CD29. In contrast, antibodies against CD18, CD49e, CD49d-FN (MoAb P4G9 blocks VLA-4 binding to the CS-1 domain of FN), CD54, and Class I MHC had no effect on adhesion of B-cell precursors to BM stromal cells.

Because the aforementioned results indicated that B-cell precursor adhesion to untreated BM stromal cells was mediated by VLA-4-VCAM-1, we repeated the experiment using IL-1β (Fig 5B) or IL-4 (Fig 5C) treated BM stromal cells to determine if the mechanism of adhesion was similar. As shown in Fig 5B, incubation of BM stromal cells with IL-1β (10 pg/mL) lead to a significant increase in B-cell precursor adhesion from 13% ± 0.6% to 26% ± 1.3% (97% increase in adhesion). Even though 10 pg/mL IL-1β upregulates both VCAM-1 and CD54 expression (Fig 3), blocking studies with MoAb yielded results similar to those in Fig 5A. This is evidenced by significant blocking of B-cell precursor adhesion using MoAb specific for CD49d-FN + V (inhibition = 79%), CD29 (inhibition = 77%), and VCAM-1 (inhibition = 74%). Inhibitory antibodies specific for CD18 and CD54 had no effect on B-cell precursor adhesion, indicating that a CD18/CD54 interaction does not mediate adhesion of B-cell pre-

cursors to BM stromal cells under these experimental conditions. In addition, antibodies specific for CD49d-FN, CD49e, and class I MHC also had no effect on adhesion. BM stromal cells treated with IL-4 (Fig 5C) gave identical results to those obtained with IL-1β treated stroma. When BM stromal cells were treated with IL-4 (10 ng/mL) so that only

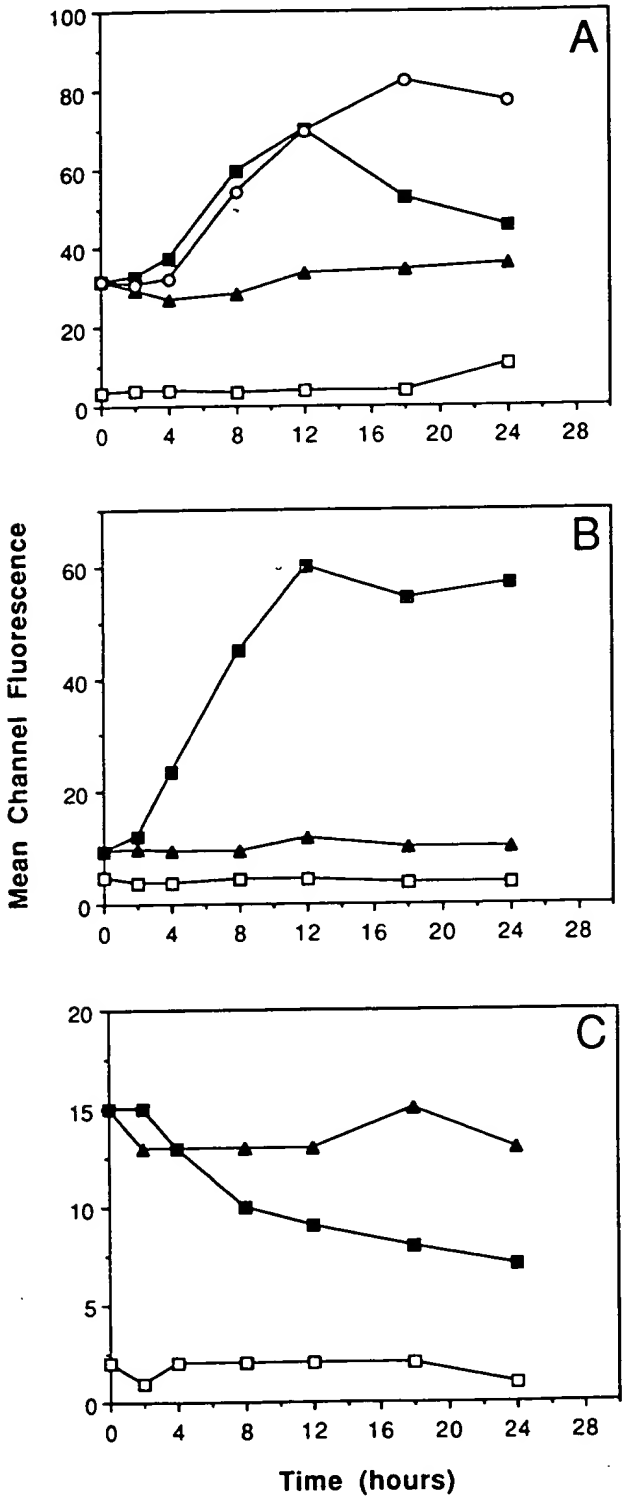

Table 1. Regulation of B-Cell Precursor/BM Stromal Cell Adhesion by IL-1β, IL-4, and TGF-β

Culture Condition	Mean Channel Fluorescence* VCAM-1	CD54	% Adhesion†
Medium alone	26	9	19 ± 0.8
IL-1β (100 pg/mL)	53	61	26 ± 0.9‡
IL-4 (10 ng/mL)	61	9	33 ± 0.9‡
TGF-β (10 ng/mL)	15	ND	14 ± 0.8‡

* Measured by indirect immunofluorescence using a FACScan. The IgG1 isotype-matched control mean channel fluorescence value for VCAM-1 was 4, and the IgG2a isotype-matched control mean channel fluorescence value for CD54 was 5. ND, not done.

† Percent adhesion was calculated as described in Materials and Methods. Values for the % adhesion represent the mean ± SE of 8 replicates.

‡ $P < .0001$ compared with medium alone.

VCAM-1 is upregulated (Fig 3C, F and Table 1). B-cell precursor adhesion increased from 13% ± 0.6% to 31% ± 1.4% (140% increase in adhesion). Once again, B-cell precursor adhesion was significantly blocked with antibodies specific for CD49d-FN + V (% inhibition = 84%), CD29 (% inhibition = 80%), and VCAM-1 (inhibition = 71%), whereas antibodies specific for CD54, CD18, CD49d-FN, CD49e, or class I MHC had no significant effect.

Phenotypic characteristics of adherent and nonadherent B-cell precursors. Ryan et al[45] reported a maturation-dependent adhesion of adult human B-cell precursors to human BM fibroblasts, with the highest adhesion by the most immature B cells ($CD10^+/CD34^+$), and adhesion decreasing steadily with each stage of maturation ($CD10^+/CD20^-$, $CD10^+/CD20^+$, $CD10^-/CD20^+$). Therefore, we investigated the maturation stage of human fetal BM B-cell precursors that adhere to BM stromal cells. Figures 6 and 7 show the expression of CD34 and κ and λ light chains on preadherent,

Fig 4. (A) Kinetics of the upregulation of BM stromal cell surface expression of VCAM-1 by IL-1β, and IL-4. BM stromal cells were incubated with medium alone (▲), IL-1β (100 pg/mL) (■), or IL-4 (10 ng/mL) (○) for 0 to 24 hours and the mean channel fluorescence of VCAM-1 was determined by indirect immunofluorescence on a FACScan. The BM stromal cells incubated with medium alone were also stained with isotype-matched IgG1 myeloma control protein (□). (B) Kinetics of the upregulation of BM stromal cell surface expression of CD54 by IL-1β. BM stromal cells were incubated with medium alone (▲) or IL-1β (100 pg/ml) (■) for 0 to 24 hours and the mean channel fluorescence of CD54 was determined by indirect immunofluorescence on a FACScan. The BM stromal cells incubated with medium alone were also stained with isotype-matched IgG2a myeloma control protein (□). (C) Kinetics of the downregulation of BM stromal cell surface expression of VCAM-1 by TGF-β. BM stromal cells were incubated with medium alone (▲) or TGF-β (1 ng/ml) (■) for 0 to 24 hours and the mean channel fluorescence of VCAM-1 was determined by indirect immunofluorescence on a FACScan. The BM stromal cells incubated with medium alone were also stained with isotype-matched IgG1 myeloma control protein (□). A, B, and C each consist of data from one representative experiment, but within each experiment all time points were simultaneously analyzed using paraformaldehyde fixed cells.

adherent, and nonadherent B-cell precursors. The adherent B-cell precursors were not enriched for $CD34^+$ cells compared with preadherent cells (Fig 6). Furthermore, although the difference was slight, there was an increase in the percentage of κ^+ and λ^+ cells in the adherent fraction (Fig 7). Identical results were obtained in an independent experiment performed on a different day with different donor fetal BM B-cell precursors.

Activation of CD29 leads to increased B-cell precursor adhesion to BM stromal cells. The interaction of VLA-4 with VCAM-1 can be regulated by the activation state of the integrin.[31,33] Therefore, we determined whether B-cell precursor adhesion to BM stromal cells could also be regulated by altering the activation state of VLA-4, using the CD29 activating antibody P4G11. Table 2 shows data from two separate experiments showing increased B-cell precursor adhesion to BM stromal cells after CD29 activation with the MoAb P4G11. In experiment 1, adhesion of B-cell precursors was increased from 42% ± 1.5% to 55% ± 1.2%. Similarly, in experiment 2, adhesion of B-cell precursors was increased from 24% ± 0.5% to 54% ± 1.8%. In both experiments, B-cell precursor adhesion to BM stromal cells was completely blocked by blocking MoAb specific for CD29, consistent with the results shown in Fig 5.

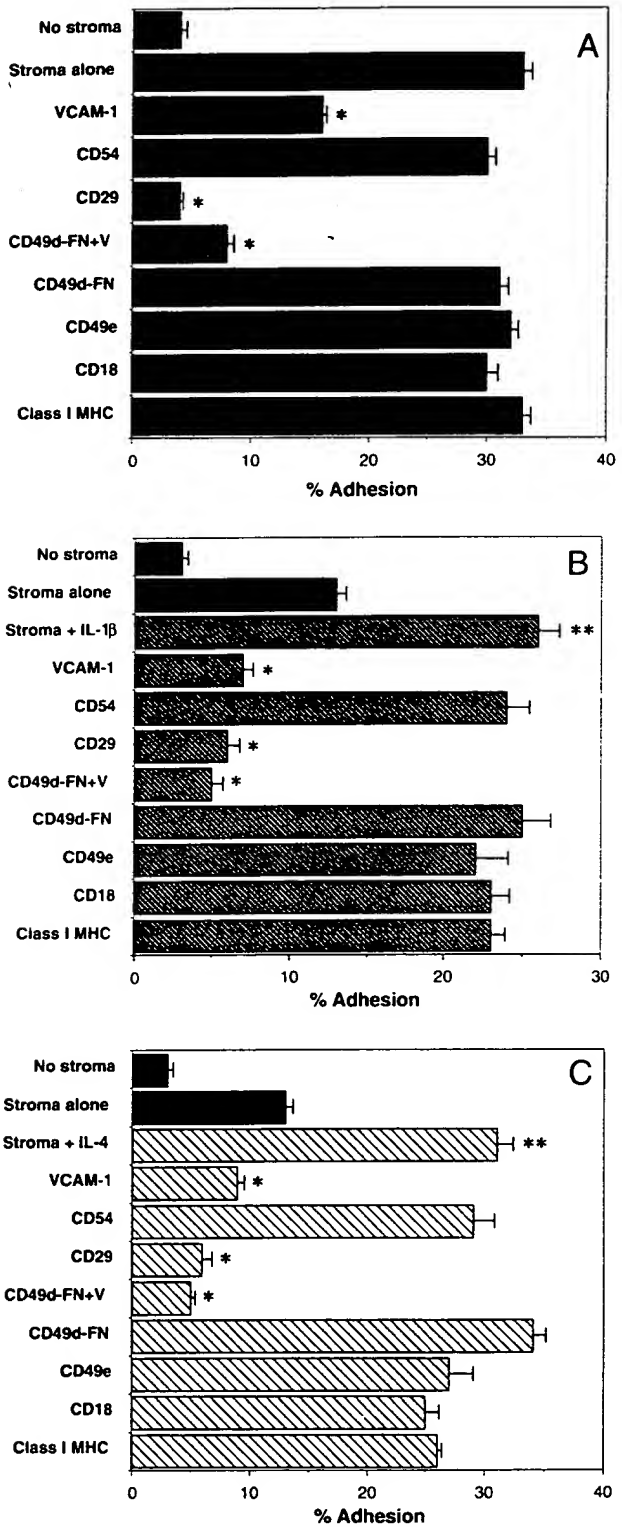

Fig 5. Blocking of B-cell precursor adhesion to (A) untreated BM stromal cells or (B) BM stromal cells treated with IL-1β, or (C) IL-4. BM stromal cells were preincubated with medium alone (■), IL-1β (10 pg/mL) (▩), or IL-4 (10 ng/mL) (▨) for 18 to 24 hours, washed twice, and saturating concentrations of antibodies specific for VCAM-1, CD18, CD29, CD49d-FN+V, CD49d-FN, CD49e, CD54, and Class I MHC were added before the addition of 50,000 ^{51}Cr-labeled B-cell precursors. Antibody P4C2 against CD49d-FN+V blocks VLA-4 binding to both VCAM-1 (V) and to the CS-1 domain of FN. Antibody P4G9 against CD49d-FN blocks VLA-4 binding to the CS-1 domain of FN only. Each bar represents the mean of the adherent cpm ± standard error of eight replicates. Percent adhesion was calculated as described in Materials and Methods. (A) *P < .0001 versus stroma alone. (B) *P < .0001 versus stroma + IL-1β; **P < .0001 versus stroma alone. (C) *P < .0001 versus stroma + IL-4; **P < .0001 versus stroma alone.

DISCUSSION

Growth and differentiation of human B-cell precursors in vitro and in vivo is characterized by their apposition to BM stromal cells.[1,2] We have developed an IL-7–dependent BM stromal cell culture that supports the growth of B-cell precursors for several weeks, and growth of the B-cell precursors occurs in close proximity to the adjacent stroma.[3] Therefore, we sought to characterize the cell surface molecules that mediate adhesion between B-cell precursors and BM stromal cells, and cytokines that may regulate this process. Several conclusions can be drawn from the results presented in this study. (1) Fetal BM B-cell precursors express CD49d (the α4 subunit of VLA-4), CD49e (the α5 subunit of VLA-5), and CD29 (the β1 subunit of VLA-4 and VLA-5). (2) BM stromal cells, which support adhesion of B-cell precursors, express higher constitutive levels of VCAM-1 than CD54 and early cultures contain a small subpopulation of vWF^+ endothelial cells, but no detectable macrophages. (3) IL-1β and IL-4 upregulate BM stromal cell VCAM-1, whereas IL-1β but not IL-4 upregulates CD54. In contrast, TGF-β decreases BM stromal cell VCAM-1. (4) The cytokine-mediated enhancement or inhibition of VCAM-1 correlates with the capacity of BM stromal cells to support the adhesion of B-cell precursors. (5) Blocking experiments using a panel of MoAb to

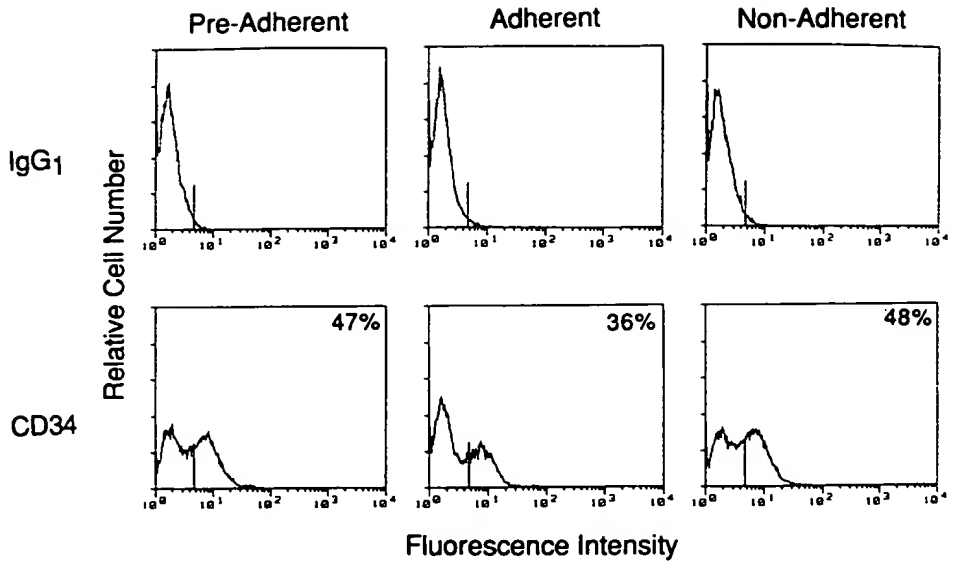

Fig 6. Expression of CD34 on preadherent, BM stromal cell adherent and BM stromal cell nonadherent B-cell precursors. CD34 expression was detected using HPCA-2-FITC. Negative control staining used IgG1-FITC.

adhesion molecules showed that B-cell precursor adhesion to untreated and cytokine-treated BM stromal cells is primarily mediated by a VLA-4-VCAM-1 interaction. Even though B-cell precursors express low levels of CD11a/CD18 and BM stromal cells express cytokine-inducible CD54, this receptor/counterreceptor complex plays little or no role in adhesion of these cells. (6) Adhesion of B-cell precursors to BM stromal cells can be enhanced by direct stimulation of B-cell precursors with anti-CD29 MoAb.

Ryan et al[21] have recently reported that VLA-4 and VCAM-1 mediate the adhesion of adult BM B-cell precursors to adult BM fibroblasts, respectively. Two other recent reports have described the VLA-4/VCAM-1-dependent adhesion of $CD34^+$ hematopoietic progenitors to BM stromal cells grown under Dexter conditions.[25,26] Our demonstration that fetal BM B-cell precursors express CD49d, CD49e, and CD29 (Fig 1), and our MoAb blocking data implicating VLA-4/VCAM-1 as the primary adhesive interaction between B-cell precursors and BM stromal cells, indicates that fetal and adult BM B-cell precursors and $CD34^+$ hematopoietic progenitors use similar adhesive mechanisms. Interestingly, no role for FN in the functional binding of VLA-4 and VLA-5 expressing

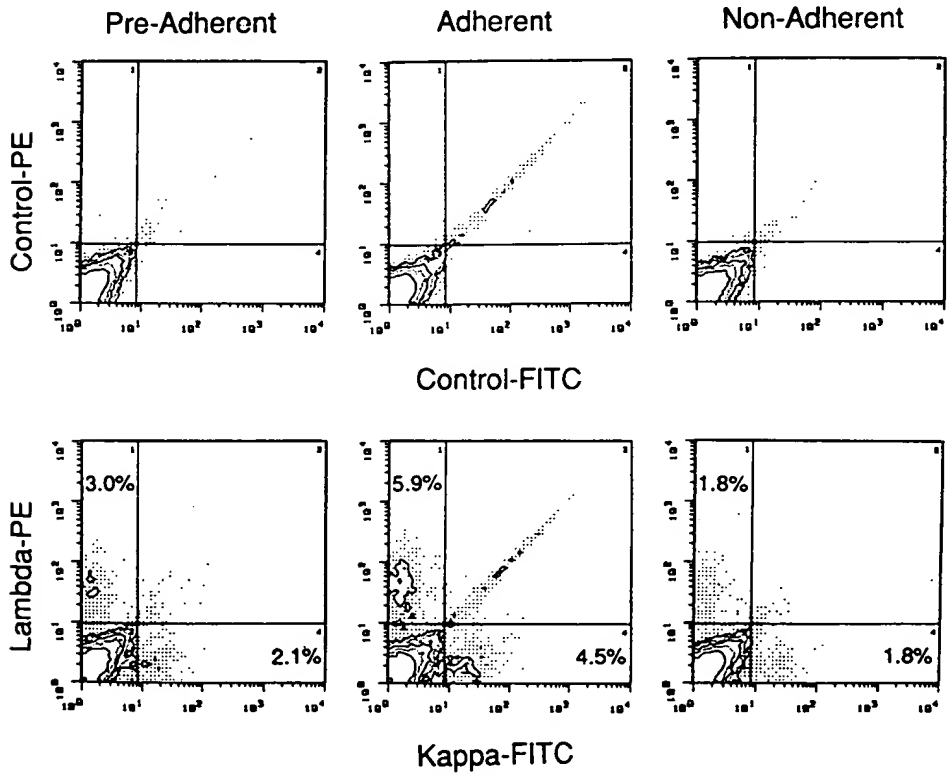

Fig 7. Expression of κ and λ light chains on preadherent, BM stromal cell adherent and BM stromal cell nonadherent B-cell precursors. κ and λ expression was detected using $F(ab')_2$ goat anti-human κ-FITC + $F(ab')_2$ goat anti-human λ-PE. Negative control staining used $F(ab')_2$ goat Ig conjugated to FITC and PE. The diagonal events in the adherent cultures probably represent dead cells.

Table 2. Adhesion of B-Cell Precursors to BM Stromal Cells After Activation With Anti-CD29

Culture Condition	% Adhesion*
Experiment 1	
No stroma	4 ± 0.4
Stroma alone	42 ± 1.5
Anti-VCAM-1	16 ± 1.0
Anti-CD54	30 ± 1.5
Anti-CD29†	55 ± 1.2
Anti-CD29‡	5 ± 0.3
Experiment 2	
No stroma	5 ± 0.3
Stroma alone	24 ± 0.5
Anti-VCAM-1	14 ± 0.3
Anti-CD54	24 ± 0.7
Anti-CD29†	54 ± 1.8
Anti-CD29‡	4 ± 0.3

* Percent adhesion was calculated as described in Materials and Methods. Values for the % adhesion represent the mean ± SE of 8 replicates.
† CD29 activating MoAb (P4G11).
‡ CD29 blocking MoAb P5D2 (experiment 1) or P4F7 (experiment 2).

B-cell precursors to BM stromal cells was detected.[21] In contrast, Teixidó et al[26] showed the adhesion of $CD34^+$ stem cells to BM stromal cells via VLA-4, VLA-5, and LFA-1-dependent mechanisms, and also described the adhesion of $CD34^+$ stem cells to recombinant soluble VCAM-1 (via VLA-4) and FN (via VLA-4 and VLA-5).[26]

Ryan et al[45] have described the maturation-dependent adhesion of adult B-lineage cells to human marrow fibroblasts, with the $CD10^+/CD34^+$ B-cell precursors exhibiting the greatest adhesion. In contrast, our studies using fetal BM showed no preferential adhesion of $CD34^+$ B-cell precursors to BM stromal cells (Fig 6). If anything, we noted a very subtle tendency toward preferential adhesion of more mature B cells expressing κ and λ light chains (Fig 7). Furthermore, we used FACS to purify $CD10^+/CD34^+$ and $CD10^+/CD34^-$ populations, and examined their adhesion to BM stromal cells. These results were entirely consistent with the data in Figs 6 and 7, and indicated slightly greater adhesion by the $CD10^+/CD34^-$ cells (B. Dittel and T. LeBien, unpublished observations). The differences in B-cell precursor adhesion between our studies and those of Ryan et al may reflect a difference in the adhesive requirements of fetal and adult BM B-lineage cells.

Studies by Miyake et al[20] have shown that adhesion between murine B-cell precursors and a cloned murine stromal cell line is mediated by the interaction of murine VLA-4 with a 100 Kd VCAM-1-like glycoprotein,[20] now known to be the murine homolog of VCAM-1.[46] Additional studies by Miyake et al[47] showed that antibodies directed against murine VLA-4 will interrupt the outgrowth of murine pre-B cells in Whitlock-Witte long-term BM cultures. Therefore, the cell surface molecules that mediate B-cell precursor/BM stromal cell adhesion appear to be similar in the two species. Studies are currently underway to directly assess the functional role of VLA-4/VCAM-1 in our IL-7–dependent B-cell precursor growth assay.

Following our initial studies, which defined the adhesive interaction between B-cell precursors and BM stromal cells, we examined the effect of cytokines on this process. This was a logical next step because cytokines secreted in the bone marrow microenvironment could conceivably influence adhesion. Cytokine-mediated regulation of VCAM-1 and CD54 has been extensively studied on human umbilical vein endothelial cells (HUVEC), and there are both similarities and differences compared with BM stromal cells. Whereas BM stromal cells express higher constitutive levels of VCAM-1 than CD54 (Fig 3), somewhat the opposite occurs on HUVEC that express little or no detectable VCAM-1 as assessed by flow cytometry[12] and enzyme-linked immunosorbent assay (ELISA).[35] Furthermore, side by side comparison indicates that resting HUVEC express higher cell surface levels of CD54 than VCAM-1.[35] BM stromal cells (Fig 3) and HUVEC[12,35] both respond to IL-1β by upregulating CD54 and VCAM-1. However, whereas BM stromal cells upregulate VCAM-1 in response to IL-4 (Figs 3 and 4), IL-4 exerts no effect on HUVEC.[35] Somewhat conversely, IL-4 had no effect on BM stromal cell CD54 (Fig 3), but a recent report indicates that IL-4 can increase CD54 on dermal fibroblasts.[48] In a recent study examining the cytokine inducibility of VCAM-1 on BM stroma cultured under Dexter conditions, Simmons et al[25] showed an increased expression of VCAM-1 on incubation with IL-1, IL-4, and TNF-α, similar to our observations on cytokine-regulated VCAM-1 expression on BM stromal cells cultured under serum-free conditions. These collective results underscore the notion that cytokine effects on expression of specific genes (in this case genes encoding adhesion molecules) can substantially vary between different cell types.

An unexpected and important discovery in our study is the TGF-β–mediated decrease in BM stromal cell surface VCAM-1. An effect of TGF-β on VCAM-1 cell surface expression has, to our knowledge, not been previously reported. The TGF-β–mediated decrease in cell surface VCAM-1 (Figs 3 and 4) correlated with a decrease in the capacity of BM stromal cells to support the adhesion of B-cell precursors (Table 1). TGF-β induced a decrease in VCAM-1 in the entire adherent BM stromal cell population (Fig 3). Nonetheless, the ubiquitous expression of TGF-β receptors[49] leaves open the possibility that TGF-β could be acting indirectly by modulating cytokine production in a subpopulation of the adherent cells. Binding of TGF-β to a variety of mesenchymal, epithelial, and lymphoid cells often leads to an enhancement of cell adhesion.[49] Specifically, TGF-β can increase the mRNA and cell surface levels of several integrin subunits.[50-53] However, TGF-β does not enhance the level of cell surface VLA-4 on B-cell precursors (B. Dittel and T. LeBien, unpublished observations). Therefore, at the level of B-cell precursor/stromal cell interactions, TGF-β may exert a relatively selective effect on the stromal cell. TGF-β is produced by many normal cells and tissues, including BM stromal cells[54,55] and B-lineage cells.[56] TGF-β is released from most cells as a latent complex unable to bind to cell surface receptors, and exposure to acidic pH or plasmin can release active TGF-β from the latent complex.[49] Therefore, it is conceivable that an acidic microenvironment in the bone marrow could generate active TGF-β, which in turn could regulate VCAM-1.

The predominant adherent cell in the BM stromal cell cultures studied herein is a fibroblast-like cell constitutively

expressing VCAM-1 and CD10 (Fig 2). However, depending on the age of the BM culture, a small subpopulation of vWF^+ putative BM endothelial cells were present (Fig 2). Because VCAM-1 is induced on HUVEC stimulated with IL-1,[12,35] it is possible that a small component of the IL-1β–enhanced adhesion of B-cell precursors is attributable to increased binding to vWF^+ cells. A surprising observation was the absence of STRO-1 (Fig 2), which is readily detectable on BM stromal cells present in human Dexter cultures.[25,26,39] This was not attributable to technical problems because we readily detected STRO-1 on glycophorin A^+ erythroid precursors. The most likely explanation for this difference is that the predominant adherent cell that constitutes a Dexter culture is distinct from the adherent cell that constitutes our serum-free BM stromal cell culture.

There is mounting evidence that integrins subserve more than just a passive role in promoting cell-cell interaction, and Hynes[4] has recently reviewed the evidence supporting a role for integrins in information transfer. For example, ligand binding to integrins leads to increased tyrosine phosphorylation and cytoplasmic alkalinization in a variety of cells.[4] Data in Table 2 show that some anti-CD29 MoAb (ie, P5D2 or P4F7) completely ablate adhesion of B-cell precursors to BM stromal cells, whereas the anti-CD29 MoAb P4G11 enhances adhesion. These results are reminiscent of recent reports showing enhanced binding of human peripheral blood lymphocytes to endothelial cells and extracellular matrix.[31,33] The favored interpretation of the results in both studies is that anti-CD29 induced a conformational change and/or increased affinity of VLA-4 for VCAM-1, leading to an increase in adhesion.

In conclusion, our results provide new insight into the regulation of human B-cell development. We hypothesize that B-cell development is regulated at the stromal cell level by specific cytokines that mediate opposing effects on the cell surface level of VCAM-1. The importance of VLA-4/VCAM-1 in the adhesion of murine and human B-cell precursors to BM stromal cells is undeniable. Enhancement of B-cell precursor growth could result from the increased adhesion of B-cell precursors that follows an increase in BM stromal cell VCAM-1. This implies that enhanced signaling through VLA-4 (potentially mimicked by anti-CD29 MoAb) may occur following an increase in the density of available VCAM-1 molecules at a B-cell precursor/BM stromal cell interface. Thus, although there may be additional BM stromal cell surface molecules that regulate B cell precursor proliferation and/or differentiation, VCAM-1 clearly plays a primary role in the adhesion of and (potentially) direct signaling to the B-cell precursor.

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Exhibit B.